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Lesions to the medial preoptic nucleus affect immediate early gene immunolabeling in brain regions involved in song control and social behavior in male European starlings

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Abstract

The medial preoptic nucleus (POM) is a brain region outside of the song control system of songbirds. It has been implicated in song production, sexual motivation, and the integration of both sensory and hormonal information with appropriate behavioral responses. The POM is well positioned neuroanatomically to interact with multiple regions involved in song, social behavior, and motivation. However, little is known about the brain regions with which the POM directly or indirectly communicates to influence song. To gain insight into the neuronal circuits normally activated in association with POM activity during male song, we compared activity within multiple brain regions using immunolabeling for protein products of immediate early genes (IEGs) *zenk* (aka *egr-1*) and *c-fos* (indirect markers of neuronal activity) in sham and POM-lesioned male European starlings (*Sturnus vulgaris*). Compared to sham lesions, POM lesions disrupted song and interest in a nest box and females responded less to POM-lesioned males. POM lesions reduced numbers of IEG-labeled cells and disrupted correlations between numbers of IEG-labeled cells and song within several song control, limbic, hypothalamic and midbrain regions. These results are consistent with the possibility that the POM integrates activity among nuclei involved in song control, social behavior, and motivational state that work in concert to promote sexually motivated communication.

Keywords

song control system; preoptic area; vocal communication; sexually motivated song; electrolytic lesion

INTRODUCTION

Converging data indicate that vocal communication in songbirds involves coordination among brain regions involved in vocal output, social behavior and motivation (e.g. (Alger and Ritters, 2006; Goodson, 1998; Goodson et al., 1999; Heimovics and Ritters, 2005, 2006, 2007; Ritters and Ball, 1999; Ritters et al., 2005; Ritters et al., 2004). The medial preoptic area is a brain region implicated in communication (Alger and Ritters, 2006; Heimovics and Ritters, 2005, 2007; Ritters and Ball, 1999; Ritters et al., 2000; Ritters et al., 2005; Ritters et al., 2004), sexual motivation (reviewed in (Hull et al., 1999; Panzica et al., 1996), and the integration of sensory and hormonal information with appropriate behavioral responses to environmental and social stimuli (reviewed in (Ball and Balthazart, 2004; Wood, 1998). Increasing evidence suggests a critical and possibly integrative role for the POM in song.

However, the brain regions with which the POM communicates to regulate song have not been identified.

Many songbird species, including European starlings (*Sturnus vulgaris*), breed seasonally. Breeding condition male starlings (i.e. males in spring-like photoperiod conditions with high testosterone) produce high levels of song, referred to here as sexually motivated song, when presented with female conspecifics (reviewed in (Eens, 1997). Evidence supports a stimulatory role for the POM in male song produced within this context. Lesions to the POM inhibit song production in male starlings exclusively within a breeding context and reduce song bout length (Alger and Ritters, 2006; Ritters and Ball, 1999), a song attribute used by females to assess male attractiveness (e.g. (Eens et al., 1991; Gentner and Hulse, 2000; Mountjoy and Lemon, 1996). Relationships between song and immediate early gene (IEG; indirect markers of neuronal activity) immunolabeling further implicate the POM in sexually motivated male song in songbirds (Heimovics and Ritters, 2005, 2007; Ritters et al., 2004). These data suggest a key role for the POM in the production and modulation of sexually motivated male song.

The POM is anatomically well situated to influence brain areas involved in both song control and social behavior (Fig. 1). It projects both directly and indirectly to nuclei of the song control system (Ritters and Alger, 2004), a network of nuclei that collectively regulate song (for review, see (Bottjer and Johnson, 1997; Brainard, 2004; Margoliash, 1997; Wild, 1997). Additionally, the POM shares reciprocal connections with a steroid-sensitive network of limbic, hypothalamic and midbrain regions involved in song, various other forms of social behavior, motivation, and reward (Goodson, 2005; Ritters and Alger, 2004).

Together, several lines of research implicate the POM in song. Neuroanatomical connections of the POM suggest this region may play a central role in the coordination of activity within multiple brain regions involved in motivation, social behavior, and song. However, the circuits influencing or influenced by the POM during song production have not yet been identified. The goal of the present study was to begin to identify neural circuits interacting with the POM to regulate song.

METHODS

We compared immunolabeling for two IEGs in POM- and sham-lesioned male starlings to highlight functional neural circuits normally influenced by POM activity during song production. IEG activation is closely associated with neuronal activity that is correlated with behaviorally relevant stimuli. However, because not every brain region expresses every IEG, the absence of labeled cells within an area cannot be interpreted as reflecting an absence of neural activity. Thus, some regions important for song may not be identified with this technique. Despite these limitations, studies involving immunolabeling for IEGs have proven to be useful tools and have provided important insight into multiple brain regions active in association with song (reviewed in (Ball et al., 2006). To strengthen our use of this technique, we examined the protein products of two IEGs, *zenk* (aka *egr-1*) and *c-fos*, which are expressed at high levels in only partially overlapping brain areas involved in song, motivation, and social behavior (e.g. (Charlier et al., 2005; Heimovics and Ritters, 2005, 2006, 2007; Ritters et al., 2004; Velho et al., 2005). The use of two indirect indicators of neural activity can potentially provide more information on brain regions regulating song than either alone.

Animals

Thirty-one male and 78 female adult starlings were captured during fall and winter of 2004 on a farm outside of Madison, Wisconsin. They were housed indoors in stainless steel cages

in single-sex groups on a light cycle reflective of the natural outside photoperiod (ranging from 9:30L to 12L) until the experiment. All experiments were in accordance with the Guidelines of the University of Wisconsin Institutional Animal Care and Use Committee and the National Institutes of Health Guidelines. All procedures were approved by the University of Wisconsin Institutional Animal Care and Use Committee.

Hormone Manipulations

All subject males were gonadectomized. Briefly, a small incision was made just anterior to the last rib and gonads were removed using forceps in males anesthetized with isoflurane, as in Alger and Ritters (2006). Males were then placed on a photoperiod of 11L:13D for at least 12 weeks, a photoperiod under which birds will become fully reproductively active in response to testosterone (T), but will not enter a state of photorefractoriness (Falk and Gwinner, 1988).

At the time of lesion surgery, subject males received two subcutaneous 14 mm silastic implants (1.47 mm i.d., 1.96 mm o.d.; Dow Corning, Midland, MI) filled for 10 mm with crystalline T (Sigma, St. Louis, MO). Males received T as in Alger and Ritters (2006) to maximize sexual behavior, promote similar T concentrations across treatments, and allow us to attribute observed effects to POM lesions independent of relationships between the POM and gonadal T. Past work shows this treatment in castrated male starlings results in T concentrations within the physiological range of breeding males for at least 8 weeks (Duffy et al., 2000).

Females to be used as behavioral stimuli were housed on 11L:13D for at least 12 weeks. These females received subcutaneous estrogen (E₂) implants to facilitate sexual interest and associated male song production. Each female received two 18 mm silastic implants (1.47 mm i.d., 1.96 mm o.d., Dow Corning) filled for 13 mm with 17-beta-estradiol (Sigma). The hormone implantation procedure was identical to that used for male T implants.

Lesion Surgery

Males were randomly assigned to the bilateral POM lesion group (n = 21) or the bilateral POM sham lesion (n = 10) group. We assigned more birds to be lesioned than to receive a sham operation because we expected that, due to natural variation in brain size among wild-caught starlings, some of the lesions would miss the POM.

Lesion methods were as previously described (Alger and Ritters, 2006). Briefly, before each surgery, the anterior-posterior and ventral-dorsal zero coordinates were taken directly from the ear bar of a small animal stereotaxic apparatus (Kopf, Tujunga, CA) fitted with a beak cone for gas anesthesia. Males anesthetized with isoflurane were placed into the stereotaxic apparatus with the beak angled at a 45° angle below the horizontal plane. The skull was opened above the POM and the lateral-medial zero coordinate was taken from the midline at the surface of the brain. An electrode was lowered to the coordinates (A = -0.5 mm, L = ±0.15 mm, V = 7.0 mm from the zero coordinate). For each lesion 0.25 mA of anodal direct current, produced by an S88 stimulator (Grass, West Warwick, RI) and held constant by a constant current unit (Grass), was passed for 20 s through the exposed tip of the electrode. For a sham procedure, the electrode remained in the brain for 20 s without the passage of current. The electrode was rapidly removed after each lesion, the skull was closed with dental cement and the skin was sutured. In order to allow for recovery and for the dissipation of any possible stimulatory effects of the electrode, one month was allowed for recovery prior to behavioral testing.

Behavioral Testing

Test Cage Set-up and Habituation—Subject males were introduced into a separate ($76 \times 76 \times 81 \text{ cm}^3$) cage alone in a sound-attenuated indoor observation room with an 11L:13D photoperiod. The room contained a nest box, two perches, and food and water *ad libitum*. A microphone was located in the room, but outside of the cage. Twenty-four hours after introduction to the cage, a stimulus female was introduced into the room (but outside of the male's cage) for 10 minutes and then into the male's cage for 10 minutes to habituate the male and encourage his future singing behavior.

Song Recording—An observer recorded songs from behind a one-way mirror using a laptop computer, M-Audio MobilePre pre-amplifier (Irwindale, CA), and Avisoft Recorder software (an automated sound-recording software; Berlin, Germany). Avisoft software was set to record and save all vocalizations longer than 10 seconds in duration. At least 24 hours after habituation of the male with a stimulus female, male song was recorded in the presence of a stimulus female outside of the male's cage. This was continued, often in multiple recording sessions, until at least 20 songs had been recorded or until 10 hours of recording had been completed, whichever came first. After each recording session, the stimulus female was placed in the cage with the male for 10 minutes to encourage future singing behavior. Stimulus females were reused multiple times during song recording sessions. Between recording sessions and after completion of song recording, subject males were housed alone in the observation cage to which they were habituated.

Songs, defined as vocalizations at least 10 s in duration with a pause no longer than 2 s, were measured for song bout length using Avisoft SASLab Pro software. Mean song bout length was determined for each male from these recordings.

Behavioral Testing for IEG Analysis—At least 24 hours after the completion of song recording, an observer placed a handful of green nest material (which males use during courtship) into the male's cage and released a novel stimulus female into the room and recorded the male's behavior for 60 minutes from behind a one-way mirror. Sexually motivated behaviors recorded included: latency to sing from the time the female was released into the room, number of times each male sang, number of nest box directed behaviors (including the number of times the male brought green nest materials to the nest box, entered the nest box, and looked in the nest box), and number of times the female landed on the male's cage (as an assay for the potency of the males' courtship signals). Contextually nonspecific behaviors (eating, drinking, beak wiping, and preening) were also recorded. Although beak wiping is a courtship behavior in some passerine species, it does not appear to have a role in courtship in European starlings. The order of males observed was randomized.

Tissue Collection

All males were sacrificed 15 minutes following the 60-minute behavioral observation period. This time course was selected based on past research indicating this to be an effective time point at which to examine relationships between both immunolabeling for ZENK (the protein product of the *zenk* gene, the avian homologue of *zif-268*, *egr-1*, *ngfi-a* and *krox-24*) and FOS (the protein product of the *c-fos* gene) and song behavior (e.g. Heimovics and Riters, 2005, 2006, 2007). Subject males were rapidly decapitated and brains were removed and fixed overnight in a 5% acrolein solution. Males were checked at this time to confirm the presence of hormone implants and to check for testicular remnants. Following acrolein fixation, brains were cryoprotected in 30% sucrose for two days, frozen with crushed dry ice, and stored at -80°C . Frozen brains were cut in coronal sections at $40 \mu\text{m}$ using a cryostat. Every third section was collected for Nissl, labeling for FOS, or

labeling for ZENK, except for the region containing POM, in which every section was collected exclusively for Nissl for the purpose of accurate lesion reconstruction. Tissue to be analyzed for lesion reconstruction was Nissl stained with Thionin.

Lesion Reconstruction

The percentage of lesion damage located within and outside the POM for each bird was quantified from the Nissl-stained tissue using a Spot camera (Diagnostic Instruments, Sterling Heights, MI) attached to a Nikon microscope (Melville, NY) and a computer. As in our previous work (Alger and Ritters, 2006), the volumes of POM, lesions inside POM and lesions outside POM were traced with a mouse using MetaVue software (Universal Imaging Corp., Downingtown, PA). Lesions were reconstructed by tracing the areas that showed obvious lesion damage. No attempt was made to identify additional necrotic tissue, so our lesion measurements were likely conservative. We estimated POM and lesion volumes by multiplying the area for each section by 0.04 mm for the section thickness and summing these volumes for the length of the nucleus.

Immunocytochemistry

Immunocytochemistry for the protein products of the IEGs *c-fos* and *zenk* was performed in two of the series of sections collected anterior and posterior to POM [i.e. sections anterior to the septopallioesencephalic tract (TSM) and posterior to the anterior commissure (AC)] using standard techniques (Heimovics and Ritters, 2005, 2007). FOS and ZENK primary antibodies were c-fos (K-25 made in rabbit, SC-253, 1:18000; Santa Cruz Biotechnology, Santa Cruz, CA) and egr-1 (C-19 made in rabbit, SC-189, 1:5000; Santa Cruz Biotechnology). The specificity of the egr-1 antibody has been validated in starlings using a preadsorption study (Sackman et al., 2002). To validate the specificity of the anti-FOS antibody in starling tissue, immunocytochemistry was performed using the brains of three photosensitive, T-implanted males. For each of these brains, half of the sections were incubated in K-25 antibody and the other half were incubated in K-25 antibody with the blocking peptide SC-253 P (1:50; Santa Cruz Biotechnology). We observed high levels of staining in the sections containing antibody alone and no staining in sections containing antibody plus peptide. For both FOS and ZENK, secondary was biotinylated goat anti-rabbit (1:250; Jackson ImmunoResearch Laboratories, West Grove, PA) and sections were incubated in AB solution (Vector ABC kit, Vector Laboratories, Burlingame, CA) followed by Vector SG as a chromagen (Vector Laboratories). Sections were mounted on gel-coated slides, dehydrated with alcohol, and coverslipped.

IEG-immunoreactive (IEG-ir) cells were counted using a Spot camera attached to a Nikon microscope and a computer using MetaVue software. Brain nuclei analyzed included song control regions [dorsomedial nucleus intercollicularis (DM), HVC (abbreviation as proper name), robust nucleus of the arcopallium (RA), Area X (proper name), lateral magnocellular nucleus of the anterior nidopallium (LMAN), and nucleus uvaeformis (Uva)] and limbic, hypothalamic, and midbrain regions implicated in social behavior, motivation, and reward [midbrain central gray (GCt), ventral tegmental area (VTA), dorsal and ventral subdivisions of caudal lateral septum (SLcd and SLcv, respectively) nucleus taeniae of the arcopallium (TnA), lateral hypothalamus (LHy), and ventromedial nucleus of the hypothalamus (VMH); Fig. 1 and 2]. For each antibody, all tissue was run in a single batch and background labeling was fairly uniform across individuals. The MetaVue autoscale function was used to calculate the correct exposure of each image as a percentage of the total range of light, thus further reducing the variation in background among individuals. Therefore, it was possible to use a single threshold to determine which cells were to be counted as labeled for each specific antibody. In all cases the threshold selected material that a blind observer agreed was specific labeling. Cell counts were made within boxes or ovals centered within each region

of interest (Fig. 2) and counts were generated by MetaVue in each of three serial sections in both hemispheres for each bird. The nuclei locations were based on Heimovics and Ritters (2007) and Goodson et al. (2005). Counts were summed for each brain region for each male. In cases of extensive tissue damage, the individual was dropped from analysis for affected brain areas.

Statistics

Data were analyzed using the statistical software program Statistica (StatSoft 2001, Tulsa, OK) and R (R Foundation, <http://www.r-project.org>). Assumptions and appropriateness of parametric statistics were checked by establishing if sample sizes were larger than 10, establishing normal distributions using Q-Q plots and Lilliefors tests, and establishing homogeneity of variance using Brown-Forsythe tests. Behaviors and numbers of FOS- and ZENK-ir cells were compared between control males and lesioned males using Student's t-tests if all parametric assumptions were met and using Mann-Whitney U tests if parametric assumptions were not met. We used Spearman Rank Correlations to assess the relationship between numbers of IEG-ir cells and measures of song.

To investigate the extent to which differences in IEG immunolabeling result from behavioral changes induced by POM lesions (i.e. secondary effects of changes in motor behavior caused by POM lesions) or represent a disruption of integrative inputs from the POM independent of motor behavior changes, we performed multiple linear regression analyses. For each IEG and each brain area studied here, the number of IEG-ir cells were entered as the response variable and lesion condition and all measured behaviors were entered as predictors into Forward, Backward and Stepwise selection procedures for variable reduction purposes. Predictor variables chosen by these procedures were plotted against the associated response variable to assess the need for appropriate variable transformations. From the resulting candidate models, models with high adjusted R^2 , randomly scattered residual plots centered at zero, and Q-Q plots nearest the 45° line were then chosen as the final models for each IEG and each brain area. These final models were examined using multiple linear regression.

Additionally, Principal Components Analyses (PCAs) were performed on correlation matrices of IEG-ir cell counts for each brain region. Separate PCAs were performed for control and for POM-lesioned males. PCA is a statistical technique applied to a large set of variables to determine which variable subsets form coherent groups that are relatively independent of one another. Variables that are correlated with one another and which are also largely independent of other variable subsets are combined into *components*. The *first principal component* is the component retaining characteristics of the data set that contribute most to its variance; the second principal component retains the second greatest; and so on. Thus, PCA can be thought of as identifying the structure in the relationships between multiple variables in a way which best explains the variance in the complete data set. PCA allows us to explore potential shifts in relationships between FOS- and ZENK-immunoreactivity in multiple brain areas that result from POM lesions, something that is impossible with traditional statistical hypothesis testing methods.

RESULTS

Lesion Reconstruction

Of the 21 males with attempted POM lesions, 12 had successful bilateral POM damage, 7 had unilateral POM damage, and 2 had damage completely outside of the POM. Ten males had successful sham operations. The POM-lesioned group discussed here consisted of only males with successful bilateral POM damage. Data from the two males with lesions

completely outside of the POM fell within the range of the data from the sham group for all behaviors measured, so they were included in the control group. This resulted in 12 males in the control group and 12 males in the POM lesion group.

The estimate of POM damage ranged from 0.9% to 13.1% (mean $\pm$ SD = 5.2 ± 4.0 , $n = 12$), and the portion of POM damage varied slightly across males (Fig. 2B–2D). Estimates of POM damage were likely conservative because no attempt was made to identify additional necrotic tissue likely surrounding the areas of obvious lesion damage. We divided the POM into three subregions: rostral (located ventral to the TSM and dorsal to the supraoptic decussation), intermediate (POM located caudal to the TSM and rostral to the AC) and caudal (just ventral to the AC). Five lesions were confined to caudal POM. Four were restricted to intermediate POM. The remaining 3 all included intermediate POM (1 included rostral + intermediate POM, 1 included intermediate + caudal POM, and 1 included all 3 subregions).

Data from individuals with lesions confined to caudal POM were compared to data from individuals with lesions including intermediate POM using Mann-Whitney U tests. The POM subregion lesioned did not have an effect on behavior or the numbers of IEG-labeled cells, with the exception of HVC (discussed below). Due to small sample sizes when males were divided by POM subregions and lack of evidence for distinct behavioral effects of intermediate versus caudal POM lesions, data from all POM-lesioned males were pooled for further analyses.

POM lesions and behavior

During song recording (which lasted up to 10 hours), POM-lesions did not appear to affect whether or not birds sang (92% of lesioned birds sang compared to 83% of control birds). However, only 50% of lesioned animals sang at least 20 songs within the 10 hours of recording compared to 75% of control animals. Recorded songs produced by males with POM lesions were shorter compared to songs produced by control males ($Z = -1.972$, $p = 0.049$; Fig. 3B).

During behavioral testing (which lasted 60 minutes), 67% of POM-lesioned birds sang compared to 83% of control birds. Compared to controls, males with POM lesions took longer to start singing ($Z = 2.098$, $p = 0.036$; Fig. 3A) and produced fewer songs ($Z = -2.083$, $p = 0.037$; Fig. 3C) after the introduction of a female. Males with POM lesions showed fewer nest box directed behaviors ($Z = -2.709$, $p = 0.007$; Fig. 3D) and females landed less frequently on the cages of POM-lesioned males compared to control males ($Z = -2.083$, $p = 0.037$; Fig. 3E). In contrast, males with POM lesions fed and drank more frequently than control males did (for feeding, control: 5.4 ± 4.0 , $n = 12$; lesioned: 10.6 ± 6.2 , $n = 12$; $t = 2.412$, $df = 22$, $p = 0.025$; for drinking, control: 5.3 ± 3.8 , $n = 12$; lesioned: 10.9 ± 7.3 , $n = 12$; $Z = 2.057$, $p = 0.040$). POM lesions did not affect preening or beak wiping.

POM lesions decreased numbers of IEG-ir cells in song, limbic, hypothalamic and midbrain nuclei

Compared to control males, POM-lesioned males had fewer FOS-ir cells in HVC ($Z = -3.077$, $p = 0.002$), Uva ($Z = -2.890$, $p = 0.004$), GCt ($t = -2.838$, $df = 19$, $p = 0.011$) and LHv ($t = -2.305$, $df = 21$, $p = 0.031$; Fig. 4A, C, E, G). POM-lesioned males also had more FOS-ir cells in LMAN compared to control males. However, this comparison included an influential outlier, which once removed, failed to show a significant difference. Additionally, POM-lesions significantly decreased the numbers of ZENK-ir cells in HVC ($t = -3.729$, $df = 21$, $p = 0.001$), Area X ($Z = -2.078$, $p = 0.038$), GCt ($Z = -2.708$, $p = 0.007$)

and SLcd ($Z = -2.521$, $p = 0.012$; Fig. 4B, D, F, H; Fig. 5A). POM lesions did not significantly affect IEG-ir cell counts in any other brain region measured.

When tested for an effect of POM subregion lesioned, ZENK-labeling was significantly reduced in HVC in individuals with lesions to caudal POM compared to males with lesions to rostral/intermediate or intermediate POM lesions ($Z = 2.402$, $p = 0.016$). No other region was affected by the subregion of POM lesioned.

POM lesions affected correlations between measures of song and numbers of IEG-ir cells in song, limbic, hypothalamic and midbrain nuclei

Correlations between IEG-ir cell counts and measures of song differed between POM-lesioned males and controls in several brain regions. The number of songs produced had a positive correlation with numbers of ZENK-ir cells in Area X ($\rho = 0.688$, $p < 0.05$), LH_y ($\rho = 0.708$, $p < 0.05$), and VMH ($\rho = 0.626$, $p < 0.05$) in control males, but not in males with POM lesions (Fig. 5; Fig 6. A, B, D). The number of FOS-ir cells in SL_{cv} ($\rho = -0.820$, $p < 0.05$) was negatively correlated with the number of songs produced in control males, but not POM-lesioned males (Fig. 6C). The numbers of FOS-ir cells in HVC ($\rho = 0.700$, $p < 0.05$) and SL_{cd} ($\rho = 0.929$, $p < 0.05$) were positively correlated with song bout length in control males, but not POM-lesioned males (Fig. 7A, C). The number of ZENK-ir cells in SL_{cd} (and $\rho = 0.786$, $p < 0.05$) was positively correlated with song bout length in control males, but not POM-lesioned males (Fig. 7D). POM-lesioned males, but not control males, showed a positive correlation between the number of FOS-ir cells in GCt ($\rho = 0.627$, $p < 0.05$) and song bout length (Fig. 7B). IEG-ir cell counts did not correlate with measures of song in POM-lesioned or control males in any other brain area measured.

Multiple linear regression analysis revealed that POM lesion condition was a significant predictor of the number of FOS-ir cells in HVC, GCt and Uva and the number of ZENK-ir cells in HVC, SL_{cd} and LH_y. All of these best-fit regression models contained behavioral predictors as well, although these behavioral predictors were not always statistically significant. In the best-fit model relating to the number of FOS-ir cells in HVC, lesion condition was the only significant variable ($F_{2,17}=13.97$, Adjusted $R^2=0.577$, $t=-3.682$, $p=0.002$), although average song length improved model fit ($t=1.729$, $p=0.102$). Lesion condition was also the only significant variable ($t=-2.707$, $p=0.017$) in the best-fit model relating to the number of ZENK-ir cells in HVC ($F_{5,14}=5.383$, Adjusted $R^2=0.536$), although average song length (transformed with natural log, $t=1.301$, $p=0.214$), the number of songs produced ($t=0.934$, $p=0.366$), feeding (transformed as a power of e , $t=-0.796$, $p=0.439$), and the number of beakwipes ($t=1.843$, $p=0.087$) all improved model fit. The number of FOS-ir cells in GCt were significantly affected by lesion condition ($F_{5,15}=10.44$, Adjusted $R^2=0.703$, $t=-6.027$, $p<0.001$), the number of songs produced ($t=-3.289$, $p=0.005$), feeding ($t=-2.223$, $p=0.042$), drinking ($t=3.128$, $p=0.007$), and preening ($t=-4.479$, $p<0.001$). The number of FOS-ir cells in Uva were significantly affected by lesion condition ($F_{2,17}=8.924$, Adjusted $R^2=0.455$, $t=-2.182$, $p=0.043$) and average song bout length ($t=2.248$, $p=0.038$). The number of ZENK-ir cells in SL_{cd} were significantly affected by lesion condition ($F_{3,10}=7.637$, Adjusted $R^2=0.605$, $t=-2.693$, $p=0.023$) and average song bout length ($t=2.729$, $p=0.021$) and the number of beakwipes improved model fit but was not significant ($t=2.037$, $p=0.069$). The number of ZENK-ir cells in LH_y were significantly affected by lesion condition ($F_{2,20}=5.433$, Adjusted $R^2=0.287$, $t=-2.505$, $p=0.021$) and preening ($t=-2.494$, $p=0.022$). Lesion condition improved the fit of models for the number of FOS-ir cells in Area X and in LMAN, but it was not significant in either case ($p=0.196$ and $p=0.413$, respectively). Lesion condition was not present in any other linear model.

POM lesions affect interactions among song, limbic, hypothalamic, and midbrain nuclei

Only birds that had both FOS and ZENK data for all brain regions analyzed were included in the PCA. The following brain regions were included: HVC, Area X, Uva, GCt, VTA, VMH, and LHy. FOS- and ZENK-ir cells in RA, DM, LMAN, SL and TnA were not included due to the lack of sufficient birds with data for all regions. This resulted in one PCA for control males (n=8) and one PCA for POM-lesioned males (n=10), each with 14 variables. To verify that data associated with the decreased sample sizes used in PCA were consistent with the complete data set, scatterplots and correlations of FOS- and ZENK-labeling with number of songs produced were created for the reduced data set. Correlation patterns were consistent with the complete data set.

The PCA performed on IEG data for control males yielded seven principal components (PCs; Table 1, Fig. 8A). Of these seven, the first two described 57.0% of the variation. Variables contributing at least 10% to the first two PCs are reported here. PC1 was largely a function of ZENK- and FOS-labeling in LHy, FOS-labeling in VMH and Area X and ZENK-labeling in GCt. The second PC was primarily composed of ZENK- and FOS-labeling in HVC, ZENK-labeling in Area X, and FOS-labeling in Uva (Table 3).

The PCA performed on IEG data for POM-lesioned males yielded nine PCs (Table 2, Fig. 8B). Of these nine, the first two described 50.4% of the variation. The first PC mostly reflected FOS- and ZENK-labeling in VMH, FOS-labeling in VTA, and ZENK-labeling in Uva. The second PC was primarily described by FOS- and ZENK-labeling in HVC, ZENK-labeling in Area X, and FOS-labeling in VTA (Table 3).

DISCUSSION

POM lesions suppressed song and other courtship behaviors and altered activity in and interactions among nuclei that work in concert to promote sexually motivated communication. These data highlight relationships between the POM and multiple brain regions involved in song production, motivation and social behavior that likely work together to regulate sexually motivated song.

POM lesions disrupt sexually motivated song and other breeding context appropriate behaviors

In contrast to control males who displayed high levels of sexually motivated song and interest in nest boxes, POM-lesioned males behaved in a manner more typical of males in a non-breeding context. Specifically, POM-lesioned males produced shorter songs than control males, a behavior characteristic of the non-breeding season when song is used for purposes other than mate attraction (Riters et al., 2000). Consistent with this finding, females landed less often on the cages of POM-lesioned males than on cages of controls, suggesting lesions to the POM reduced the attractiveness to females of male song or other male attributes. In addition to these novel findings, our data confirmed past studies showing that relative to control males, POM-lesioned males produce fewer songs, take longer to initiate singing, and interact less frequently with a nest box in response to a female (Alger and Riters, 2006; Riters and Ball, 1999). Behavioral deficits were specific to sexually motivated behaviors, indicating that reductions in song and interest in a nest box produced by lesions did not represent global behavioral deficits.

IEG data suggest that the POM communicates with multiple regions to influence song

POM lesions disrupt activity in the song control system—Despite extensive overlap in song production (Fig. 6), POM-lesioned males had fewer IEG-labeled cells in Area X compared to controls. This suggests that differences in POM-lesioned and control

males are not simply due to differences in the amount of song produced by the two groups but may be attributed to fundamental differences in the neural circuitry underlying song. (This is the case for Area X and additionally LH_y, VMH and SL, discussed below). Similar to the mammalian basal ganglia, Area X appears to be part of a generalized pathway for sensorimotor learning, including song learning, in songbirds (reviewed in (Doupe et al., 2005; Margoliash, 1997; Person et al., 2008)). Area X also exhibits differential activity depending on the social context in which song is produced (Hessler and Doupe, 1999; Jarvis and Nottebohm, 1997; Jarvis et al., 1998; Ritters et al., 2004; Sasaki et al., 2006). Previous evidence shows that Area X activity is negatively related to song directed towards a conspecific in adult male zebra finches (Hessler and Doupe, 1999; Jarvis et al., 1998; Sasaki et al., 2006) and presumed sexually motivated song in male house sparrows (Ritters et al., 2004). In contrast to these findings, in the present study the numbers of ZENK-ir cells in Area X of control males *positively* correlated with song production. Given the role of Area X in song learning, differences between this study and previous ones could relate to species differences in sensorimotor aspects of song learning between closed-ended learners (e.g. zebra finches and house sparrows) and open-ended learners (e.g. starlings). Alternatively, these differences could result from the fact that starlings do not sing directed and undirected song in as obvious a manner as do zebra finches and thus cannot be quantified in the same manner. Future studies are required to explore these ideas.

HVC is a song control region involved in motor output (reviewed in (Fee et al., 2004; Margoliash, 1997)) that can be influenced by indirect projections from POM to DM, GCt, and VTA (Fig. 1; (Appeltants et al., 2000; Bottjer et al., 1989; Ritters and Alger, 2004)). HVC is implicated in the regulation of song bout length in starlings and other songbirds (e.g. (Bernard et al., 1996; DeVoogd et al., 1993)), and here lesions to the POM abolished the positive correlation between FOS-ir and song bout length that was observed in control animals. This deficit along with the reduction of IEG-ir cells in HVC induced by POM-lesions may reflect a reduction in motor-driven IEG-expression, given that POM-lesioned males sang fewer and shorter songs than controls. However, the linear regression analysis suggests that this is not the case. The presence of POM lesions was the only significant predictor of the numbers of both FOS-ir and ZENK-ir cells in HVC, indicating that the effect of POM lesions on IEG-labeling in HVC was independent of behavioral differences. Interestingly, the precise rostral-caudal location of POM lesions affected HVC labeling, with caudal lesions more substantially reducing IEG-labeling compared to more rostral lesions. These results are consistent with past studies indicating that the POM consists of functionally distinct subdivisions (reviewed in (Balthazart and Ball, 2007)).

The POM can also influence the motor pathway of the song control system through indirect projections to Uva (Bottjer et al., 1989; Ritters and Alger, 2004; Striedter and Vu, 1998; Williams and Vicario, 1993; Fig. 1). Similar to HVC, Uva exhibits singing-induced IEG expression (Jarvis et al., 1998), suggesting the reduction in FOS-ir in Uva caused by POM lesions could also be a motor-driven effect resulting from the decreased song production in lesioned males. In support of this interpretation, both the presence of POM lesions and average song bout length were significant predictors of the number of FOS-ir cells in Uva. Uva is involved in interhemispheric coordination of song (reviewed in (Margoliash, 1997; Schmidt et al., 2004)) and the integration of visual and auditory information (reviewed in (Margoliash, 1997; Wild, 1997)). Thus, the disruption of activity in Uva resulting from lesions to the POM may underlie the failure of POM-lesioned males to sing long song appropriate within a courtship context.

POM lesions disrupt activity in limbic, hypothalamic and midbrain regions—

The POM, GCt, VMH, and SL are considered components of a “social behavior network”, a reciprocally connected set of steroid sensitive nuclei proposed to underlie multiple forms of

social behavior (Goodson, 2005; Newman, 1999). Although not proposed as part of this network, LH_y is another region implicated in social behavior that is reciprocally connected with the POM (Riters and Alger, 2004). In the present study, POM lesions affected IEG-labeling within each of these regions.

Although the precise role of the septum in song is not known, septal modulation of male song is strongly associated with social context (e.g. (Goodson, 1998; Goodson et al., 1999; Heimovics and Riters, 2006, 2007). Past work in starlings shows correlations between IEG-labeling in SL_{cv} and song production to be negative in males singing sexually motivated song (Heimovics and Riters, 2006, 2007). Consistent with these results, we found a negative correlation between song and FOS-labeling in SL_{cv} in control males. POM lesions thus appeared to abolish this relationship. Perhaps damage to the POM disrupted the normal involvement of the septum in context appropriate song, causing lesioned males to sing in a manner uncharacteristic of the breeding season. In the present study, IEG quantification was also taken within another subdivision of SL, SL_{cd}. Both the presence of POM lesions and average song bout length were significant predictors of the number of ZENK-ir cells in SL_{cd}. POM lesions significantly decreased IEG-labeling in SL_{cd} and eliminated IEG correlations with song bout length in SL_{cd}. Interestingly, in contrast to SL_{cv}, IEG-labeling in SL_{cd} correlated *positively* with song, consistent with several studies indicating that the septum consists of functionally distinct subdivisions (Goodson et al., 2004; Goodson et al., 2005; Heimovics and Riters, 2006, 2007). These results are consistent with data suggesting a complex, possibly subdivision specific role for the septum in song regulation that may differ depending upon social context. The precise role of the septum and its subregions in song must be clarified in future studies.

POM lesions additionally reduced FOS-ir cell counts in LH_y and eliminated the positive correlations between ZENK-labeling in VMH and LH_y and song production. Both the presence of POM lesions and the number of preening events were significant predictors of the number of ZENK-ir cells in LH_y, suggesting that the effects of POM lesions on ZENK-immunolabeling is not completely independent of behavior. Both LH_y and VMH are hypothalamic regions that respond to sexually salient cues (e.g. (Orsini et al., 1985). Furthermore, LH_y activation may be reinforcing (reviewed in (Wise, 1996). Thus, by affecting activity in VMH and LH_y, POM lesions may disrupt sexual salience or reward associated with seeing a female or the act of singing itself. Interestingly, previous work showed a positive correlation between the number of ZENK-labeled cells in VMH and song production in breeding condition male starlings but not non-breeding condition males (Heimovics and Riters, 2007). So in this way, POM-lesioned breeding condition male starlings again appear to be similar to non-breeding condition males.

POM-lesioned males had fewer IEG-ir cells in GCt compared to controls. GCt (the avian homolog of mammalian periaqueductal gray) is implicated in sexual behavior (e.g. (Charlier et al., 2005; Floody and DeBold, 2004; Heimovics and Riters, 2007; Marson, 2004) and vocal communication in multiple species (reviewed in (Jurgens, 1994). Interestingly, FOS-labeling in GCt was positively correlated with song bout length in POM-lesioned animals, but not in control animals. Although difficult to interpret, this result could reflect a compensatory response of GCt to POM lesions. Furthermore, the presence of POM lesions was found to contribute significantly to the number of FOS-ir cells in GCt; however, behavioral measures including the number of times the bird sang, fed, drank and preened, were also significant predictors. This suggests complex interrelationships between POM lesions, behavior and IEG-labeling in GCt.

POM lesions alter relationships between song and social behavior nuclei—
POM lesions affected how song and social behavior nuclei work together as a circuit, as

indicated by different first and second principal components in POM-lesioned males compared to controls (Table 3). Furthermore, males with and without POM lesions had different association patterns among IEG-immunoreactivity in various brain regions when plotted on these PCs. When IEG-ir cell counts for each brain area in controls were graphed by the first two PCs, all song control nuclei grouped together and nuclei involved in social behavior formed a separate group, suggesting separate physiological associations in activity (Fig. 8). This pattern was lost in POM-lesioned males, suggesting that interfering with normal POM function disrupts song production by disrupting relationships among multiple brain regions.

Conclusion

Our results indicate that the POM interacts with a number of song production, limbic, hypothalamic and midbrain circuits during the production of sexually motivated song and nest box interactions. Lesions to the POM may thus hinder sexually motivated song through impedance of interhemispheric coordination and motor aspects of song production, motivation and reward associated with song production, and integration of external social context information with the animal's internal state.

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ABBREVIATIONS

AC	anterior commissure
Area X	proper name
DLM	medial nucleus of the dorsolateral thalamus
DM	dorsomedial nucleus intercollicularis
DSV	ventral supraoptic decussation
GCt	midbrain central gray
HVC	abbreviation as proper name
IEG	immediate early gene
LHy	lateral hypothalamus
LMAN	lateral magnocellular nucleus of the anterior nidopallium
nCPa	nucleus commissurae pallii
Nif	nucleus interface of the nidopallium
NIII	oculomotor nerve
nXIIts	tracheosyringeal portion of the hypoglossal nucleus
POM	medial preoptic nucleus
PVN	paraventricular nucleus
RA	robust nucleus of the arcopallium

Ram/rVRG	nucleus retroambigialis/rostral ventral respiratory group
Rt	nucleus rotundus
SL	lateral septal nucleus
SLcd	dorsal subdivision of caudal lateral septum
SLcv	ventral subdivision of caudal lateral septum
TnA	nucleus taeniae of the arcopallium
TSM	septo pallio-mesencephalic tract
Uva	nucleus uvaeformis
vIII	third ventricle
VMH	ventromedial nucleus of the hypothalamus
VTA	ventral tegmental area

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A. POM Direct Connection with the Song Control Motor Pathway

B. POM Indirect Connections with the Song Control Anterior Forebrain and Motor Pathways

C. POM Connections with Brain Regions Involved in Social Behavior

Figure 1.

Sagittal schematics showing functionally distinct pathways by which the POM possibly influences sexually motivated song production via A) a direct connection with the song control motor pathway, B) indirect connections with the song control anterior forebrain and motor pathways and C) connections with nuclei involved in social behavior.

Figure 2. POM lesion damage and immunocytochemistry regions. A, E–G) Boxes/ovals represent areas in which cFOS- and ZENK-labeled cells were counted. Region areas (mm^2) are indicated in parentheses. B–D) Reconstruction of POM lesion damage in Nissl stained tissue, cumulative across all 12 POM-lesioned subjects. Light gray ovals represent lesion damage located in one bird. Darker gray ovals represent lesioned areas common to at least 2 males.

Figure 3.

Histograms illustrating effects of POM lesions on measures of mean A) song latency, B) song bout length, C) numbers of songs produced, D) next box directed behaviors and E) number of female landings on the subject males' cages. Asterix indicates $p < 0.05$.

Figure 4.

Histograms illustrating mean numbers of IEG-ir cells in sham-lesioned and POM-lesioned males for A) FOS-ir in HVC, B) ZENK-ir in HVC, C) FOS-ir in Uva, D) ZENK-ir in Area X, E) FOS-ir in GCT, F) ZENK-ir in GCT, G) FOS-ir in LHy and H) ZENK-ir in SLcd. Asterix indicates $p < 0.05$.

Figure 5. Photomicrographs at 100X of representative IEG-immunolabeling. Both FOS- and ZENK-ir were similar in appearance. This figure shows representative ZENK-immunoreactivity in A) Area X and B) VMH. For each brain area, top panels are from control birds and bottom panels are from birds with POM lesions. Also for each brain area, left panels are from birds producing no song and right panels are from birds with high song output (This control bird sang 52 songs and this lesioned bird sang 49 songs). The scale bar indicates 0.5 mm.

Figure 6.

Scatterplots illustrating relationships between numbers of IEG-labeled cells and numbers of song produced in sham-lesioned and POM-lesioned males for A) ZENK-ir in Area X, B) ZENK-ir in LHv, C) FOS-ir in SLcv and D) ZENK-ir in VMH. Each dot represents data for a single individual. Missing data points indicate tissue lost due to damage. Solid lines indicate lines of best fit for significant correlations.

Correlations Between IEG-immunoreactivity and Song Bout Length

Figure 7.

Scatterplots illustrating relationships between numbers of IEG-labeled cells and song bout length in sham-lesioned and POM-lesioned males for A) FOS-ir in HVC, B) FOS-ir in GCt, C) FOS-ir in SLcd and D) ZENK-ir in SLcd. Each dot represents data for a single individual. Missing data points indicate tissue lost due to damage. Solid lines indicate lines of best fit for significant correlations.

Figure 8.
Illustration of PCA results: A) Projection of IEG-labeling on the factor plane for PC1 and PC2 for control animals. B) Projection of IEG-labeling on the factor plane for PC1 and PC2 for POM-lesioned animals.

Table 1

The loadings and cumulative percentage of variation explained by each of the PCs obtained by PCA of FOS- and ZENK-labeled cells in seven brain regions in control males. Numbers in bold indicate variables contributing at least 10% to the first two PCs.

Variables	Principal Component Loadings						
	PC1	PC2	PC3	PC4	PC5	PC6	PC7
HVC FOS	-0.102	-0.599	-0.487	-0.617	0.003	0.091	-0.070
HVC ZENK	0.309	-0.807	-0.467	0.099	-0.063	0.136	-0.050
Area X FOS	0.749	-0.215	-0.465	0.301	-0.005	-0.294	0.018
Area X ZENK	-0.424	-0.725	0.194	-0.232	-0.392	-0.213	-0.067
Uva FOS	0.652	-0.573	-0.419	-0.180	-0.037	-0.159	0.111
Uva ZENK	-0.135	-0.416	0.765	-0.321	0.062	0.311	0.140
GCl FOS	-0.439	0.472	0.084	-0.208	-0.715	-0.151	-0.004
GCl ZENK	-0.729	-0.355	-0.168	0.540	-0.099	0.057	0.095
VTA FOS	-0.262	0.559	-0.678	-0.248	-0.116	-0.073	0.281
VTA ZENK	-0.660	-0.251	-0.426	0.349	-0.307	0.321	0.034
VMH FOS	-0.789	0.241	-0.402	-0.081	0.188	-0.076	-0.331
VMH ZENK	-0.541	-0.423	0.566	0.114	0.155	-0.405	0.081
LHy FOS	-0.737	0.128	-0.428	-0.219	0.440	-0.028	0.125
LHy ZENK	-0.889	-0.387	-0.035	0.044	0.195	-0.125	0.054
Cumulative % of Variation	34.047	56.979	77.079	86.058	93.790	98.084	100.00

Table 2

The loadings and cumulative percentage of variation explained by each of the PCs obtained by PCA of FOS- and ZENK-labeled cells in seven brain regions in POM-lesioned males. Numbers in bold indicate variables contributing at least 10% to the first two PCs.

Variables	Principal Component Loadings								
	PC1	PC2	PC3	PC4	PC5	PC6	PC7	PC8	PC9
HVC FOS	0.254	0.870	0.389	0.016	0.044	-0.111	-0.082	-0.084	0.006
HVC ZENK	-0.026	0.784	-0.487	-0.267	0.051	0.121	0.012	0.233	0.063
Area X FOS	0.382	-0.188	0.583	-0.474	0.198	-0.226	0.371	0.160	-0.023
Area X ZENK	-0.167	0.763	-0.179	0.124	0.048	-0.560	0.007	-0.163	-0.017
Uva FOS	0.395	0.211	0.808	0.070	0.296	-0.071	-0.144	0.164	-0.035
Uva ZENK	-0.721	0.357	0.231	-0.044	0.386	0.313	-0.209	-0.033	-0.070
GCl FOS	0.613	0.545	-0.391	0.360	-0.131	0.058	0.128	0.088	0.005
GCl ZENK	0.068	-0.246	0.361	0.834	-0.302	-0.039	0.058	0.108	-0.031
VTA FOS	0.723	-0.093	0.501	0.195	0.331	0.144	-0.024	-0.176	0.129
VTA ZENK	-0.490	0.643	0.370	0.312	0.004	0.242	0.223	0.064	0.006
VMH FOS	-0.968	-0.085	0.021	0.065	0.024	-0.096	0.168	0.062	0.091
VMH ZENK	-0.885	-0.155	0.270	0.137	0.218	-0.093	0.194	-0.078	0.009
LHy FOS	-0.416	0.016	0.619	-0.180	-0.489	-0.161	-0.360	0.116	0.058
LHy ZENK	0.069	0.360	0.506	-0.363	-0.581	0.239	0.222	-0.183	-0.020
Cumulative % of Variation	28.274	50.433	70.793	81.265	89.309	94.160	97.900	99.708	100.00

Table 3

Summary of correlated associations between IEG-labeling in POM-lesioned and control animals based on PCA. Brain regions are listed in the order of the strength of their influence on the associated PC.

	Control Males	POM Lesion Males
Factors influencing PC1 with $\geq 10\%$ variable contribution	LHy, VMH, Area X, GCt	VMH, VTA, Uva
Factors influencing PC2 with $\geq 10\%$ variable contribution	HVC, Area X, Uva	HVC, Area X, VTA